



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. : 09/806,178
Applicants : Nobuo Nagai et al.
Filed : June 18, 2001
Title : USE OF COMPOUNDS THAT REDUCE ALPHA2-
ANTIPLASMIN IN VIVO FOR THE PREPARATION
OF A COMPOSITION FOR THE TREATMENT
OF ISCHEMIC STROKE
Group Art Unit : 1647
Examiner : Christopher J. Nichols

DECLARATION

Commissioner for Patents
P.O. Box 1450
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I, Désiré José Collen, as an expert in the field of molecular biology, and, generally, in the field of the above-identified patent application, hereby declare:

1. That I am an expert in the field of the present invention due to my experience in the field of molecular biology, as evidenced by my attached *curriculum vitae* and through my position as Director of the research laboratory in which the inventors and myself in the above-identified application conducted the research disclosed in the application.

2. I have read and am thoroughly familiar with the contents of the above-identified patent application, of which I am a co-inventor, as well as of the prior art references cited in the application.

3. Plasminogen is an inactive plasma protein that, under limited conditions, is converted to the active protein plasmin, a proteolytic enzyme which is responsible for clot lysis by digesting fibrin and destroying many other clotting

factors. After a clot is formed, plasminogen can absorb into the clot, but clot lysis will not occur until plasminogen is activated into plasmin by a plasminogen activator, such as tissue plasminogen activator, which also absorbs into the clot. This plasminogen activation therefore occurs locally only, at the site of the clot, and only trace amounts of plasmin are generated even locally. Thus, systemic administration of plasminogen will never result in a measurable amount of circulating plasmin.

4. The plasma protein α_2 -antiplasmin (α_2 -AP) is a plasmin inhibitor which rapidly forms a very stable 1:1 stoichiometric enzymatically inactive complex with plasmin. Plasmin is composed of two chains, the A chain and the B chain, with each chain containing a different binding site. The A chain contains a lysine binding site and the B chain contains a catalytic active site. It is the B chain which contains the catalytic active site that binds to α_2 -AP, forming a stable inactive complex with the reactive site of α_2 -AP. The resulting steric hindrance of α_2 -AP binding to the active site of plasmin effectively neutralizes α_2 -AP activity. (The A chain, with its lysine binding site, binds predominantly to fibrin). Therefore, systemic administration of an effective dose of plasmin will effectively neutralize circulating α_2 -AP. Additionally, α_2 -AP like fibrin, has a site that is complementary to the lysine binding site, of plasmin(ogen). Thus when α_2 -AP is depleted the lysine binding site of plasmin preferentially binds to fibrin, resulting in greater fibrinolysis.

5. Two other plasmin species, miniplasmin and microplasmin, have similar catalytic domains as plasmin and thus display the same binding characteristics to α_2 -AP. In particular, miniplasmin contains the one kringle domain of plasmin but lacks the lysine binding site, and microplasmin lacks all five kringle domain of plasmin as well as the lysine binding site, but both contain the active site for α_2 -AP. Thus, miniplasmin and microplasmin are truncated forms of plasmin which lack the

lysine binding site, but have the catalytically active site that neutralizes α_2 -AP activity. Additionally, because they both lack the lysine binding site, miniplasmin and microplasmin react more slowly with α_2 -AP, and thus remain longer in the circulation, thus have a longer half-life (3-4 sec) in the circulation as compared to plasmin (about 0.1 sec), the latter of which binds more rapidly to α_2 -AP and to fibrin.

6. Plasminogen and lys-plasminogen have a lysine binding site but not the active catalytic site of plasmin. Thus, plasminogen and its derivatives, such as lys-plasminogen, are incapable of neutralizing circulating α_2 -AP; although plasminogen and lys-plasminogen may bind to an inconsequential degree to the complementary lysine binding site of α_2 -AP, neutralization of α_2 -AP does not result. Furthermore, because plasminogen activation occurs locally at the site of a clot, as described above, with only trace amounts of plasmin generated thereon, systemic administration of plasminogen or its derivatives does not and cannot result in circulating levels of plasmin that are capable of neutralizing α_2 -AP. The only effect that circulating plasminogen or its derivatives would have would be to slow somewhat the reaction time between plasmin, miniplasmin, microplasmin, etc. with α_2 -AP, by binding with the lysine binding site of α_2 -AP, but nonetheless never neutralizing α_2 -AP.

7. The above conclusions have been supported by the following investigation conducted by myself in collaboration with my colleagues. *Nagai N. et al., Recombinant human microplasmin: production and potential therapeutic properties. J. of Thrombosis and Haemostasis 2003; 1: 307-313.*

Introduction

It has previously been reported that infarct size is reduced in mice with a permanent ligation of the middle cerebral artery (MCA) when plasmin is

administered, and that this reduction is associated with neutralization of circulating α_2 -AP. Nagai N. et al. *Depletion of Circulating α_2 -antiplasmin by Intravenous Plasmin or Immunoneutralization reduces focal cerebral ischemic injury in the absence of arterial recanalization. Blood 2001; 97: 3086-92.* To further substantiate the effect of neutralization of α_2 -AP and reduction of infarct size, the effects of plasmin, microplasmin, and microplasminogen in ischemic stroke models in mice were examined in the following investigation.

Method

Focal cerebral ischemia was produced by permanent occlusion of the MCA in mice. Briefly, mice were anesthetized with isoflurane and the left temporal muscle was transected and the skull exposed. A 1 mm opening was made in the region over the MCA, and the MCA was occluded by ligation with thread. Microplasmin, microplasminogen, plasmin, tissue plasminogen activator (TPA) or solvent was given intravenously as a bolus, from 15 min up to 6 h after ligation of the MCA. After 24 h, 3 days or 7 days, the animals were sacrificed and decapitated. The brain was sectioned and the sections were differentially stained so that the necrotic infarct area remains unstained and distinguishable from stained viable tissue. The focal cerebral ischemic infarct size was determined as the sum of the unstained areas of the sections, multiplied with their thickness.

Results

Bolus injection of microplasmin decreased α_2 -AP proportionally to the microplasmin dose, which recovered partially within 2 h and fully within 24 h, suggesting that α_2 -AP depletion was transient during the first hours after the injection of microplasmin. Bolus injection of 4 mg/kg TPA caused minor α_2 -AP reduction.

Ligation of the MCA induced a cerebral infarct with a volume of approximately 29 mm³. Injection of 2.5 mg/kg microplasmin had no significant effect on infarct size, whereas 5.0 mg/kg, or 5.0 mg/kg followed 45 min later by another 2.5 mg/kg, produced a 15% reduction of infarct size. These results are in line with the transient partial reduction of α_2 -AP with the lower dose and the more persistent depletion with 5.0 mg/kg microplasmin. Infarct size was reduced with microplasmin injections up to 4 h after MCA occlusion, and when measured up to 3 days after microplasmin administration.

In contrast, administration of 40 mg/kg of microplasminogen or 4.0 mg/kg of TPA did not reduce infarct size.

Conclusion

In mice with a MCA ligation, an intravenous bolus of 5.0 mg/kg of microplasmin reduced infarct size approximately 15%, whereas 40 mg/kg of microplasminogen had no effect. The decrease in infarct size is correlated with the neutralization of circulating α_2 -AP. I therefore conclude that the results of this study demonstrate that the inability of circulating plasminogens and their derivatives, such as microplasminogen, to affect infarct size is due to their inability to irreversibly bind to, and therefore neutralize, circulating α_2 -AP. Furthermore, because plasminogen activation occurs locally at the site of a clot, with only trace amounts of plasmin generated thereon, systemic administration of plasminogen or its derivatives, such as lys-plasminogen, cannot generate circulating levels of plasmin high enough to react stoichiometrically with α_2 -AP to effect its neutralization. Plasminogen and its derivatives, therefore, have no inherent capability of binding to circulating α_2 -AP because the conversion of plasminogen to plasmin occurs only locally at the site of the clot or infarct (the extrasystemic compartment), and not systemically (the systemic

compartment). Thus, the fact that plasmin is found only in the extrasystemic compartment upon its activation makes it impossible for plasmin generated therein to neutralize α_2 -AP present in the systemic compartment. The only effect that plasminogen and its derivatives have is to affect the reaction time between plasmin, miniplasmin, microplasmin, or any other truncated form of protein comprised of the plasmin catalytic active site, with α_2 -AP, by virtue of its binding with the lysine binding site of α_2 -AP.

8. There is approximately 5 L of blood plasma containing approximately 350 mg of α_2 -AP. Because α_2 -AP reacts stoichiometrically with plasmin (MW 70,000), the effective dosage amount of plasmin that would need to be administered systemically in order to neutralize 100% of α_2 -AP is about 5 mg/kg body weight. Because microplasmin is approximately 60% smaller than plasmin by weight, the effective dose needed for the same neutralization effect by microplasmin is about 2 mg/kg body weight. (Neutralization of 50% α_2 -AP would require one-half as much). Thus, administration of plasmin, microplasmin or miniplasmin in a dosage amount ranging between 1.5 to 7.0 mg/kg body weight would effectively neutralize 100% of circulating α_2 -AP, resulting in significant reduction in infarct size. These dosage amounts would not be learned by one skilled in the art by the Eibl reference, because Eibl discloses only dosage amounts for lys-plasminogen and neither addresses nor suggests what plasmin dosages need to be systemically administered for any purpose. Moreover, even if one could convert the dosage amounts for lys-plasminogen to a comparable plasmin dosage, because lys-plasminogen activation occurs only locally at the site of the infarct *in vivo*, producing only trace amounts of plasmin, any conversion amount calculated would be irrelevant for determining the dosage amount needed to achieve the circulating plasmin needed to neutralize α_2 -AP.

9. It would not take undue experimentation for one skilled in the art to determine other protein variants which have the active catalytic site of plasmin. In fact, techniques for developing proteins or polypeptides having the active catalytic domain of plasmin, such as miniplasmin and microplasmin, have been available for years. For example, it is well known that elastase digestion of plasmin yields two fragments: angiostatin, formed by kringle 1-4, and miniplasmin, composed of kringle 5 and the serine proteinase domain, i.e., the catalytic active site domain of plasmin. Production of midi-plasmin, a plasmin that lacks kringle 1-3 and thus is composed of kringle 4 and 5, having the serine proteinase domain of plasmin, has been available as early as 1995. *Christensen, S. et al. Biochem. J. 1995: 305(Pt1): 97-102.* Moreover, the generation of miniplasmin composed of kringle 5 and having the serine proteinase catalytic active site has been known since 1979. *Christensen, L. et al. Biochim. Biophys. Acta 1979: 567: 472-481.* Therefore, developing compounds having the catalytically active site of plasmin has been well known in the art for several decades and production of these compounds is a well known technology in the art that does not require any undue experimentation.


Furthermore, all treatments employing thrombolytic compounds inherently contain a certain degree of risk for internal bleeding, and the associated risks are well known. For many patients suffering from, for example, cerebral ischemic infarcts, however, the small degree of risk of treatment is significantly better than the very real alternative risk of death.

10. I have read the Eibl et al. reference and am familiar with its contents. Eibl et al. discloses a method for treating ischemia, infarction and the reperfusion injury that follows ischemia by administering a pharmaceutical composition comprising lys-plasminogen. By contrast, my invention as claimed is directed to a

method for treating focal cerebral ischemic infarcts by administering systemically an effective dose of a compound capable of neutralizing circulating α_2 -AP activity and concentration, so that the size of the focal cerebral infarct is reduced. Compounds of the invention that are capable of such α_2 -AP neutralization are plasmin, miniplasmin, microplasmin, monoclonal antibodies and derivatives thereof, such as Fab fragments and scFv fragments. Eibl et al. neither teach nor suggest the subject matter of the claims because administration of a pharmaceutical composition of lys-plasminogen is incapable of binding to, and therefore neutralizing, circulating α_2 -AP, thus their composition cannot reduce the size of focal cerebral infarcts. This is because plasminogen activation occurs only locally at the site of a clot, with only trace amounts of plasmin generated even locally, and the systemic administration of plasminogen or its derivatives, such as lys-plasminogen, which is taught by Eibl et al., cannot generate circulating levels of plasmin high enough to react stoichiometrically with α_2 -AP to effect its neutralization. Plasminogen and its derivatives, therefore, have no inherent capability of binding to circulating α_2 -AP because the conversion of plasminogen to plasmin occurs only locally at the site of the clot or infarct (the extrasystemic compartment), and not systemically (the systemic compartment). This spatial segregation of endogenous moieties in an organism is known as compartmentalization, a concept that makes any inherency argument with regard to plasminogen activation leading to circulating levels of plasmin impossible, because such activation, if it happens at all, occurs only locally and not systemically.

11. I declare further that all statements made herein of my own knowledge are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.



Désire José Collen
Date 28 JULY 2003